Survival of *Pseudomonas syringae* pv. *actinidiae* on the orchard floor over winter

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Abstract *Pseudomonas syringae* pv. *actinidiae* (Psa) was first identified on kiwifruit (*Actinidia* spp.) in New Zealand in November 2010, and has since caused serious losses, particularly in Te Puke. The role of fallen leaves and pruning debris in the life-cycle of Psa in New Zealand is unknown. Survival of Psa was investigated in fallen leaves and cane prunings over winter 2011. Heavily infected leaves, and cane prunings with Psa die-back symptoms, were kept on the orchard floor and in the laboratory, and assayed weekly for the presence of viable Psa. At leaf-fall, all leaves yielded live Psa. Although detection frequency declined over time, especially after 5-6 weeks, Psa was still isolated from leaf litter 15 weeks after leaf-fall and cane prunings 11 weeks after winter pruning. These results indicate that the pathogen overwinters readily in leaf litter and pruning debris, representing a potential inoculum source for infection of new spring growth.

Keywords *Pseudomonas syringae* pv. *actinidiae*, Psa, bacterial canker, inoculum survival, qPCR.

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), the causative agent of bacterial canker disease, was first identified on kiwifruit (*Actinidia* spp.) in New Zealand in November 2010 (Everett et al. 2011) and has since caused serious vine losses, particularly in the Te Puke area. Originally found in a restricted area of Te Puke, the virulent strain of the pathogen (Psa-V) is now known to occur throughout the Bay of Plenty (Te Puke, Tauranga, Katikati, Waihi, Whakatane and Opotiki) and in one area west of Pukekohe (Auckland region) (KVH 2012).

In New Zealand, bacterial canker of kiwifruit is characterised by leaf spots (with or without a yellow halo), cane die-back, cankers and the production of orange-red or milky-white exudates. Under conditions of high humidity, bacterial exudates can also form on the underside of leaf spots. This leaf exudate has also been observed in Japan (Serizawa et al. 1989).

Scortichini et al. (2012) noted that bacterial exudates can disperse a large amount of inoculum within and between orchards. The presence of heavily colonised fallen leaves and pruned canes on the orchard floor at the beginning of winter, and potentially significant volumes of bacteria associated with those tissues, raises questions about the role of the fallen leaves and cane prunings in the life-cycle of Psa.

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Currently, pruned canes are dropped to the orchard floor, mulched and left to decompose naturally over winter. The survival of Psa in this material has not been studied. This work was designed to determine the role of fallen leaves and cane prunings in the over-wintering of the pathogen, thereby allowing for more informed management decisions by growers.

MATERIALS AND METHODS

Heavily infected leaves were harvested from a population of *Actinidia chinensis* seedlings in Block 52 of the Te Puke Research Orchard (TPRO) during leaf-fall (25 May 2011). A large proportion of the leaves had conspicuous bacterial exudates on the lower surface. The leaves were held in four gauze-enclosed frames on the orchard floor, under symptomless 'Hayward' kiwifruit vines at TPRO. Four sets of leaf material were also held in the laboratory at ca 20°C, under conditions of alternating wet and dry (night/day). Leaf samples were taken at weekly intervals over the period 25 May to 9 September 2011.

Prunings from 'die-back' canes of *A. chinensis* were cut into 10 cm lengths during winter pruning (14 July 2011). These were set up in a further four frames on the orchard floor, adjacent to the leaf frames. Four sets of cane material were also held in the laboratory under the same conditions as the leaves. Thirteen sets of cane samples were taken, approximately weekly, from day 0 through to 13 weeks (15 July–17 October 2011). At each weekly sampling, isolations were made from each of the four sets of plant material in the field and in the laboratory, providing four replicates of each treatment.

Bacterial isolations

Bacterial isolations were made as follows: pieces of plant tissue (4 × 4 cm areas of leaf or 5 mm lengths of cane) were aseptically excised and macerated in 2 ml bacteriological saline (BS) (0.85% NaCl in sterile distilled water), and left for at least 5 min. A 200 µl aliquot of the resulting suspension was then streaked onto King's B medium (King et al. 1954). Plates were incubated at room temperature (ca 20°C) for 2 days.

DNA extraction

BS (1 ml) was added to the mixed bacterial cultures on each plate and the washings vortexed thoroughly. An aliquot (100 µl) of each of the suspensions was added to 900 µl BS and centrifuged at 8500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 ml BS and centrifuged at 8500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 ml EDTA (1 mM). An aliquot (200 µl) of the final suspension was then boiled at 100°C in a water bath for 5 min, cooled on ice, then stored at -20°C until further use. The resultant DNA sample was diluted 2.5-fold for Polymerase Chain Reaction (PCR) preparation.

qPCR analysis

qPCR was performed using a Roche Lightcycler® 480 and the Psa-specific primers F3 and R4 described by Rees-George et al. (2010). In addition, bacterial 23S primers (Anthony et al. 2000) were used in qPCR to check the quality of the DNA.

Each 10 µl reaction contained: 2.5 µl template, 1.5 µl water, 0.5 µl 5 µM PsaF3 primer (or 0.5 µl 5 µM 23SF), 0.5 µl 5 µM PsaR4 primer (or 0.5 µl 5 µM 23SR), and 5 µl SYBR Green Master 1 (Roche). All wells were sealed and then centrifuged at 1000 rpm for 1 min at 20°C before amplification.

The qPCR run consisted of a pre-amplification cycle at 95°C for 10 min, followed by 45 amplification cycles of 95°C for 5 s (denaturation), 65°C for 7 s (primer annealing) and 72°C for 7 s (extension). After amplification, melt-curve analysis was performed by heating the samples to 95°C for 5 s and cooling down to 65°C for 1 min, followed by a gradual increase to 97°C with five to ten acquisitions per cycle. This was followed by a cooling cycle at 40°C for 10 s.

Each assay included a positive control (*P. syringae* pv. *actinidiae* isolate cc691 from New Zealand *Actinidia* sp.) and negative controls (BS, and *P. marginalis* isolate cc678 and *P. syringae* pv. *syringae* isolate cc726, both from New Zealand *Actinidia* sp.).

In this study, a Cp (crossing point or threshold value) value below 30 was interpreted as a Psa-positive result and a Cp value above 35 as a negative result.

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RESULTS

Leaf litter

The frequency of recovery of viable Psa from four sets of leaf debris over the period of the experiment is shown in Figure 1. At the time of leaf-fall, all leaves tested were positive for live Psa. Although the frequency of detection declined over time, most markedly after 5–6 weeks, it was still possible to isolate Psa from both the leaf litter held on the orchard floor and in the laboratory on 9 September 2011, 15 weeks after leaf-fall.

Winter pruned canes

The frequency of recovery of viable Psa from four sets of pruned canes over the period of the experiment is shown in Figure 2. At the time of pruning, half the cane samples tested positive for viable Psa. Retrieval from cane samples tended to be intermittent over the trial period and the frequency declined over time. Nevertheless, viable Psa was recovered from the orchard and laboratory-held cane boxes on 3 October 2011, 11 weeks after pruning.

DISCUSSION

Italian researchers have previously observed that I2-Psa (Psa-V) survived for more than 45 days in infected *A. chinensis* twigs that were held in the laboratory (Marcelletti et al. 2011). This work was designed to determine whether Psa is able to survive in fallen leaves and cane prunings on the orchard floor over winter in New Zealand.

It was found that, in Te Puke, Psa survived in the leaf litter on the orchard floor for at least 3 months, by which time the leaves were largely decomposed. It survived in the cane prunings for at least 11 weeks. The more intermittent detection of Psa from the cane prunings mirrors the discontinuous distribution of Psa within live infected canes (J.L. Tyson, unpublished data).

The leaves and cane material held in orchard frames were exposed to natural orchard conditions, and were therefore potentially exposed to further inoculum from the vines within the block, or to inoculum from further afield. The results show good agreement between recoveries made from the orchard material and

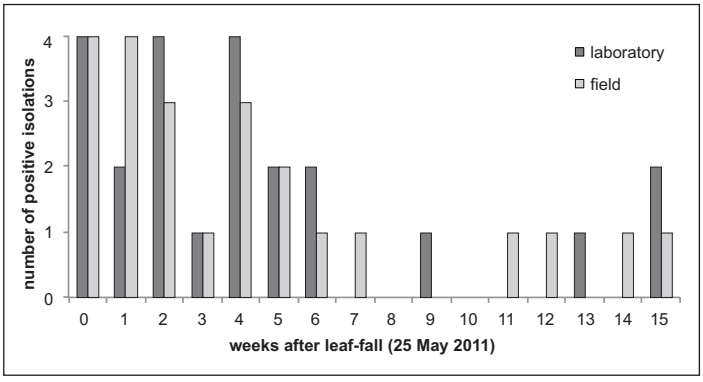

Figure 1 Frequency of isolation of viable *Pseudomonas syringae* pv. *actinidiae* (Psa) from four separate samples of kiwifruit leaf litter held in the orchard and in the laboratory over the period 25 May–1 September 2011.

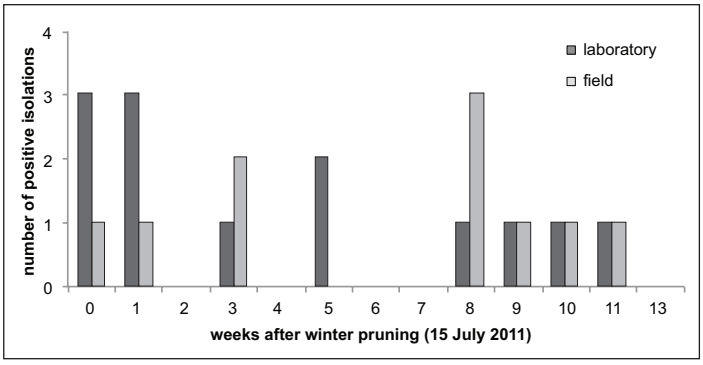

Figure 2 Frequency of isolation of viable *Pseudomonas syringae* pv. *actinidiae* (Psa) from four separate samples of symptomatic kiwifruit cane material held in the orchard and in the laboratory over the period 15 July–3 October 2011.

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that held in the laboratory where no further inoculum was possible. This allows us to be confident that they were bacteria derived from the initial infections that were being detected at each weekly sampling date.

The enrichment step in this work, in which the bacterium was isolated and grown on artificial media, demonstrated that the DNA being detected was from viable bacterial cells, not merely DNA from dead bacterial cells.

This work has shown that the plant debris from leaf-fall and winter cane prunings harboured viable Psa until well after budburst in the following spring, particularly for early cultivars such as 'Hort16A'. Therefore the debris and prunings should be regarded as potential sources of early-season inoculum within the orchard.

This is the first time that the winter carryover of the pathogen in plant debris has been identified as a potential inoculum source in the spring. Management options to reduce that risk that need investigation include the potential for 'digester' products to promote faster decomposition of debris and thus reduce Psa survival times, and whether debris size affects Psa survival. Greater knowledge of these factors will allow the development of management strategies to minimise the risk posed by infected leaf litter and pruning debris over winter and spring.

ACKNOWLEDGEMENTS

This work was funded by ZESPRI® Group Ltd/ KVH. We would also like to thank Michelle Vergara, Ngaire Larsen and Cathy McKenna for technical help and sample collection.

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